Simultaneous Analysis of Benzphetamine and Its Metabolites, and Quantitation of Urinary *p*-Hydroxy-*N*-benzylamphetamine by Micellar Electrokinetic Chromatography

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We developed a method for simultaneous analysis of benzphetamine (BZ) and its metabolites, *p*-hydroxy-*N*-benzylamphetamine (*p*HBA), *p*-hydroxybenzphetamine (*p*HBZ), amphetamine (AP), methamphetamine and *p*-hydroxymethamphetamine by micellar electrokinetic chromatography (MEKC). Urine samples from 0–15 h (3-h intervals) after oral administration of BZ (10 mg) were hydrolyzed with β -glucuronidase (EC 3.2.1.31) at 37 °C overnight. The treated urine was applied to a solid phase extraction column Bond Elut Certify[®]. After sequentially washing the column with water, 0.1 mol/l acetic acid and methanol, the samples were eluted with dichloromethane : isopropanol : 28% ammonium hydroxide = 78.4 : 19.6 : 2.0 (v/v %). The eluate was evaporated and the residue dissolved in running buffer was analyzed by MEKC. In urine from 0–3 h, AP, *p*HBZ and *p*HBA were detected. After that, only *p*HBA, which is one of the major metabolites of BZ in human urine, could be detected in the urine by the present method. A method for quantitation of *p*HBA by MEKC is described here. The effects of acetonitrile and sodium dodecyl sulfate in the running buffer of MEKC on the separation of BZ and its metabolites are also reported.

Key words capillary electrophoresis; micellar electrokinetic chromatography; benzphetamine

Drug abuse is a serious and growing problem, and the simultaneous screening and confirmation of drugs of abuse in body fluids is important for the investigation of intoxicants, the detection of potential drug users, and the control of drug addicts following withdrawal therapy. Several analytical methods have been applied to measure illegal drugs, such as gas chromatography (GC),¹⁾ gas chromatography-mass spectrometry (GC/MS)^{2,3)} and high-performance liquid chromatography (HPLC).^{4,5)} All of these methods are versatile tools, but GC and GC/MS are limited to volatile compounds, and HPLC requires gradient elution to separate many compounds and therefore HPLC analysis takes a long time.

Capillary electrophoresis is probably the most rapid analytical technique developed in recent years. Micellar electrokinetic chromatography (MEKC) allows the separation of both neutral and charged molecules in a single run and is useful for the analysis of therapeutic and illegal drugs for pharmacological-pharmaceutical, clinical and forensic purposes.

Benzphetamine (BZ) is a benzyl analog of methamphetamine (MA) and is used as an anorexic drug in the U.S.A., while in Japan its use is prohibited. BZ is metabolized in humans to *p*-hydroxybenzphetamine (*p*HBZ) and then to *p*-hydroxy-*N*-benzylamphetamine (*p*HBA), and also to MA and then to amphetamine (AP). It has also been reported that both *p*HBA and *p*HBZ are further metabolized to their corresponding glucuronides.⁶⁾ We recently reported a method for determination of the conjugated forms of BZ metabolites in human urine, *p*-hydroxy-*N*-benzylamphetamine glucuronide (*p*HBAg) and *p*-hydroxybenzphetamine glucuronide (*p*HBZg), using HPLC.⁷⁾ However, this method could not analyze AP and MA, which are also excreted in human urine after oral administration of BZ.

In this study, we describe the simultaneous analysis of BZ and its metabolites *p*HBA, *p*HBZ, AP, MA and *p*-hydroxy-

methamphetamine (*p*HMA) by MEKC, and the application of this procedure to the quantitation of *p*HBA in human urine after oral administration of BZ. In addition, the effects of acetonitrile and sodium dodecyl sulfate (SDS) in the running buffer on the migration time on MEKC are reported.

MATERIALS AND METHODS

Material The materials used in this study were purchased from the following sources: amphetamine sulfate, Takeda Pharmaceutical Industries (Osaka, Japan); methamphetamine hydrochloride, Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan); 2-phenylethylamine (PEA) and sodium tetraborate, Wako Pure Chemical Industries (Osaka, Japan); sodium dihydrogen phosphate and acetonitrile, Nacalai Tesque, Inc. (Kyoto, Japan); SDS, Fluka Chemie AG (Buchs, Switzerland); Bond Elut Certify[®] (disposable mixed-mode bonded phase silica cartridge, 130 mg) column, Varian Sample Preparation Products (Harbor, CA, U.S.A.); β -glucuronidase (EC 3.2.1.31 from *E. Coli*), Boehringer Mannheim GmbH (Mannheim, Germany). BZ, *p*HBA and *p*HBZ were prepared as described previously.⁷⁾ *p*HMA was synthesized according to the procedure of Buzas and Dufour.⁸⁾ Water was purified with a Milli-Q system (Nippon Millipore, Ltd., Tokyo, Japan).

Subjects Two healthy adult volunteers were orally administered 10 mg of BZ (benzphetamine hydrochloride). Urine was collected at 3-h intervals for 15 h and stored –30 °C until analysis. A control urine sample was collected from each subject immediately before drug dosing. The project was approved by the Kobe Pharmaceutical University Committee for Human Investigation, and volunteers were included only after informed consent was obtained in accordance with the principles of the Declaration of Helsinki.

MEKC Standard Analytical Conditions MEKC was

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performed on an Otsuka Electronic (Hirakata, Japan) model CAPI-3000 equipped with a photodiode array detector. A fused silica capillary (500 mm, 75 μm i.d., 375 mm to the detector) was used at a constant voltage of 13 kV for the separation of samples. The temperature of the capillary column was controlled at 25 $^{\circ}\text{C}$, and the sample was injected from the anode hydrostatically (20 mm, 10 s). Migrated samples were detected by on-column measurement of UV absorption with a photodiode array detector in the range of 200–300 nm. The MEKC running buffer consisted of 20 mM sodium tetraborate–sodium dihydrogen phosphate buffer, pH 8.0, containing 50 mM SDS and 15% acetonitrile.

Treatment of Urine Samples for MEKC Hydrolysis of the glucuronide of BZ and its metabolites was performed by adding 2 ml of urine samples, 2 ml of 0.1 mol/l phosphate buffer (pH 7.0) and 25 μl of β -glucuronidase solution. The mixtures were incubated at 37 $^{\circ}\text{C}$ overnight with stirring. Then, 1 μg of PEA in methanol (100 μl) was added as an internal standard. Extraction and clean-up of the hydrolyzed urine samples were carried out on a Bond Elut Certify[®] column. The extraction column was preconditioned with 4 ml of methanol and 6 ml of 0.067 mol/l phosphate buffer (pH 5.6). The hydrolyzed samples were applied to the preconditioned column, which was then rinsed with 1 ml of water, 2 ml of 0.1 mol/l acetic acid and then 4 ml of methanol. The samples were eluted with 2 ml of dichloromethane : isopropanol : 28% ammonium hydroxide = 78.4 : 19.6 : 2.0 (v/v/v). To the eluate was added 50 μl of 0.1 mol/l acetic acid to prevent loss of the volatile AP and MA during the evaporation step. The residue was dissolved in 100 μl of MEKC running buffer (20 mM sodium tetraborate–sodium dihydrogen phosphate buffer, pH 8.0, containing 50 mM SDS and 15% acetonitrile). The samples were passed through a membrane filter (pore size 0.5 μm , Millex-LH[®], Nippon Millipore, Ltd., Tokyo, Japan) and injected hydrostatically into the column (20 mm, 10 s).

Calibration Curve for *p*HBA Serially diluted authentic *p*HBA dissolved in methanol was added to tubes containing 1 μg of PEA in methanol as an internal standard. The solvent was evaporated to dryness under vacuum and the residue was dissolved in 100 μl of the running buffer. Analytical conditions were the same as described above. The concentrations of *p*HBA were determined by comparing the peak area obtained from the sample and authentic standard to that obtained from the internal standard. The concentrations were converted to those of *p*HBA by multiplying by 1.73 (the ratio of molecular weight of *p*HBA and *p*HBA).

RESULTS AND DISCUSSIONS

Effects of Acetonitrile Concentration in the Running Buffer on Migration Time To improve the resolution of the analytes in MEKC, many organic modifiers such as methanol,^{9,10} tetrahydrofuran,⁹ acetonitrile^{10,11} and dimethylsulfoxide^{12,13} have been added to the running buffer. The addition of an organic modifier to the running buffer markedly improves the resolution by increasing the peak capacity and by decreasing the micelle-water partition coefficient. Here, we investigated the effects of acetonitrile on the separation of BZ and its metabolites.

BZ and its metabolites were analyzed in running buffer containing acetonitrile at various concentrations (Fig. 1). The

Fig. 1. Effects of Acetonitrile Concentration in the Running Buffer on the Migration Time

Authentic BZ, its metabolites and PEA (25 $\mu\text{g}/\text{ml}$) were dissolved in running buffer (20 mM sodium tetraborate–sodium dihydrogen phosphate buffer, pH 8.0, containing 50 mM SDS and 15% acetonitrile) and injected hydrostatically (20 mm, 10 s). Conditions except the acetonitrile concentration were the same as those described in Materials and Methods (standard analytical conditions). —○—, electroosmotic flow (EOF); —□—, *p*HMA; —▲—, PEA; —◆—, AP; —●—, MA; —▼—, *p*HBA; —■—, *p*HBZ; —●—, BZ.

migration times increased in the presence of 5% acetonitrile (*p*HMA), 10% acetonitrile (PEA, AP), 15% acetonitrile (MA, *p*HBA, *p*HBZ) and 20% acetonitrile (BZ), and then decreased at higher concentrations. That is, an inflection point was observed at 5% for *p*HMA, 10% for PEA, AP, 15% for MA, *p*HBA, *p*HBZ and 20% for BZ. Thus, the changes in the solubility of BZ and its metabolites between the micelles and the aqueous phase according to the acetonitrile concentration should be considered. At low concentrations of acetonitrile, BZ and its metabolites interacted strongly with SDS micelles. The electroosmotic flow decreases because the zeta potential decreases with increasing acetonitrile concentration. On the other hand, the solubility of BZ and its metabolites in the aqueous phase containing 5–20% acetonitrile increased, so that BZ and its metabolites spent more time in the aqueous phase containing acetonitrile than the micelles. These changes of the partition coefficient of BZ and its metabolites between the aqueous phase containing acetonitrile and the micelles may also be caused by changing the size and shape of micelles due to increases in acetonitrile concentration.¹¹⁾

Effects of SDS Concentration in the Running Buffer on Migration Time SDS micelles are the most common surfactant used in MEKC. Therefore, we investigated the effects of SDS concentration on the migration times of BZ and its metabolites (Fig. 2). The migration times increased as the SDS concentration increased. This tendency was marked for hydrophobic compounds such as *p*HBZ and BZ. This result clearly indicated that BZ and its metabolites interacted strongly with SDS micelles with increasing degree of hydrophobicity, and that the micellar solubilization contributes to separation of the analytes.

Simultaneous Analysis of Authentic BZ and Its Metabolites by MEKC Authentic BZ, its metabolites (*p*HBA, *p*HBZ, AP, MA and *p*HMA) and PEA were dissolved in running buffer (25 $\mu\text{g}/\text{ml}$), and applied to MEKC as described above. As shown in Fig. 3, they were completely separated within 30 min. The intra-assay ($n=5$) precision of the migration time and the inter-assay ($n=5$) for the above compounds

Fig. 2. Effects of SDS Concentration in the Running Buffer on the Migration Time

Authentic BZ, its metabolites and PEA (25 $\mu\text{g/ml}$) were dissolved in running buffer (20 mM sodium tetraborate–sodium dihydrogen phosphate buffer, pH 8.0, containing 50 mM SDS and 15% acetonitrile) and injected hydrostatically (20 mm, 10 s). Conditions except the SDS concentration were the same as those described in Materials and Methods (standard analytical conditions). —○—, EOF; —□—, pHMA; —▲—, PEA; —◆—, AP; —●—, MA; —▼—, pHBA; —■—, pHBZ; —●—, BZ.

Fig. 3. Separation of Authentic BZ and Its Metabolites by MEKC

Authentic BZ, its metabolites and PEA (25 $\mu\text{g/ml}$) were dissolved in running buffer (20 mM sodium tetraborate–sodium dihydrogen phosphate buffer, pH 8.0, containing 50 mM SDS and 15% acetonitrile) and injected hydrostatically (20 mm, 10 s) under the conditions described in Materials and Methods (standard analytical conditions). Peak identification: 1, EOF; 2, pHMA; 3, PEA; 4, AP; 5, MA; 6, pHBA; 7, pHBZ; 8, BZ.

were between 0.82–1.4% and 1.4–2.9%, respectively. The precision of the peak area was improved by correcting the peak area of each analytes relative to an internal standard (PEA) and the results were between 4.8–15.3% (intra-assay, $n=5$) and 2.7–7.5% (inter-assay, $n=5$). The time required for analysis for the present method was shorter than for HPLC (55 min for pHBA and pHBZ).⁷⁾

Analysis of Urine Samples by MEKC Urine samples were treated and analyzed by MEKC as described in the Methods section. In the urine samples obtained from 0–3 h after oral administration of BZ (10 mg), AP, pHBZ and pHBA were detected by the present method (data not shown). However, BZ and other BZ metabolites were not detected from any urine samples collected. Only pHBA was observed in the urine obtained from 0–15 h (3-h interval collections) after BZ administration. This is because of the low dose oral administration of BZ (10 mg).

Calibration Curve for pHBA The calibration curve for pHBA showed good linearity between 0.15 to 1.5 $\mu\text{g/ml}$ urine and the curve passed through the zero point ($r=0.999$).

Fig. 4. A Typical Pherogram of Urine Sample from a Healthy Subject (A) after Oral Administration of Benzphetamine Hydrochloride

Two-milliliter urine samples were prepared as described in Materials and Methods. The extracted and hydrolyzed samples were dissolved in 100 μl of running buffer (20 mM sodium tetraborate–sodium dihydrogen phosphate buffer, pH 8.0, containing 50 mM SDS and 15% acetonitrile). The samples were injected hydrostatically (20 mm, 10 s) under the conditions described in Materials and Methods (standard analytical conditions). Peak identification: 1, PEA; 2, pHBA.

Table 1. Concentrations of pHBAG in Urine Samples from Subjects A and B

	0–3 h	3–6 h	6–9 h	9–12 h	12–15 h ($\mu\text{g/ml}$)
Subject A	0.85	1.37	0.71	0.52	0.33
Subject B	1.54	1.33	0.36	0.42	0.61

Urine samples were collected for 0–15 h (3-h intervals) after oral administration of BZ (10 mg). Urine samples were processed by MEKC as described in the Materials and Methods section. The concentrations were converted to those of pHBAG by multiplying by 1.73 (the ratio of molecular weight of pHBAG and pHBA).

Recovery Test of pHBA from Urine Samples To investigate the recovery of pHBA from urine samples, 200 μl of methanol solution containing 1.5 μg of pHBA and 1.0 μg of PEA was placed in tubes, then the solvent was evaporated and 2 ml of control urine was added. Each urine sample was processed as described above. The recovery was calculated as follows; concentration of pHBA observed/concentration of pHBA added $\times 100$ (%). The recovery of pHBA was $93.8 \pm 2.1\%$ ($n=5$).

Quantitation of pHBA in Urine Samples by MEKC After oral administration of BZ (10 mg) to two healthy human subjects, urine samples were processed by MEKC as described in the Methods section. Figure 4 shows a typical pherogram of urine sample (subject A) collected during 3–6 h. pHBA was detected in this urine and was identified by spiking with authentic pHBA, comparison of the UV spectrum (200–300 nm) and migration time. The limit of detection for pHBA was about 30 pg/injection (0.15 $\mu\text{g/ml}$ urine). The peak for pHBA was not detected in 0–15 h urine samples (subjects A and B), when these samples were not treated with β -glucuronidase. Moreover, this peak was not observed in the control urine samples (subjects A and B). These data suggest that pHBA in urine after oral administration of BZ is present in the form of glucuronide.

The concentrations of pHBA in urine samples obtained from the above subjects were measured by the present method. The concentrations were converted to those of pHBAG by multiplying by 1.73 (the ratio of molecular weight of pHBAG and pHBA) (Table 1). The levels of

pHBAG were compared with the results obtained by HPLC described elsewhere⁷⁾ and a good correlation coefficient was observed ($n=10$, $y=1.18x-0.45$, $r=0.977$).

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